REMARKS

The September 16, 2005 Official Action and the references cited therein have been carefully reviewed. In light of the amendments presented herewith and the following remarks, favorable reconsideration and allowance of the application are respectfully requested

At the outset, it is noted that the Examiner has maintained the Restriction Requirement set forth in the papers mailed on March 28, 2005 and July 18, 2005 and made it final.

At page 3 of the Office Action, the Examiner has objected to the specification for containing embedded hyperlinks and/or other form of browser-executable code. In response, the specification has been amended to delete the embedded hyperlinks on pages 83 and 84, thereby rendering the objection moot. No new matter has been introduced into the specification by the foregoing amendment.

The Examiner has objected to 1) claim 2 for allegedly containing a typographic error; 2) claim 29 for allegedly reading on a non-elected protein and combination of proteins; and 3) claim 53 for allegedly depending from a canceled claim. In response, claim 2 has been amended to change "the agent is selected it if changes" to "the agent is selected if it changes"; and claim 53 has been amended to depend from claim 1. These claim amendments serve to render the objections to claims 2 and 53 moot. Additionally, Applicants respectfully request the examination of currently amended claim 53 on its merits.

At page 4 of the Office Action, the Examiner has rejected claim 1-7, 14-25, 27-32, and 52 under 35 U.S.C. §112, first paragraph. It is the Examiner's position that that subject matter encompassed by these claims is not enabled by the specification.

The Examiner also has rejected claim 15 under 35 U.S.C. §112, second paragraph for alleged indefiniteness.

At pages 8-13, the Examiner has rejected claims 1-5, 14, 16-20, 22, 33, and 52 under 35 U.S.C. §102(b) as allegedly anticipated by Wang et al. (British Journal of Pharmacology (1997)122,1405-1410). Claims 1, 6, 7, 18, 22, 23, and 52 stand rejected under 35 U.S.C. §102(b) as allegedly anticipated by Stephens et al. (Endocrine Reviews Vol. 16, No. 4, 2005).

Finally, at page 13 of the Office Action, the Examiner has rejected claims 1-7, 14, 16-20, 22, 23, 27-33, and 52 under 35 U.S.C. 103(a) as allegedly unpatentable over either Wang et al. or Stephens et al. in view of U.S. Patent No. 5,744,300 to Linskens et al. (hereafter patent '300).

The foregoing constitutes the entirety of the objections and rejections raised in the September 16, 2005 Official Action. In light of the present claim amendments and the following remarks, each of the above-noted rejections under 35 U.S.C. §§ 112, first and second paragraphs, 102(b) and 103(a) is respectfully traversed.

1. Amendments to the Claims:

Claim 1 has been amended by specifying three specific requirements which the differentially expressed proteins must satisfy in step (b). Support for these amendments can be found between pages 20 and 25 of the specification. Requirement (i) described at page 21, lines 26-29. The protein should be differentially expressed between insulin resistant (i.e. affected) subjects (i.e., the first biological sample), and normal or comparatively insulin sensitive subjects (i.e., the second biological sample). Requirement (ii) is supported by the disclosure at page 22, lines 16-24. The protein should be differentially expressed between affected individuals who have

been treated with a known effective drug or other treatment (i.e., the third biological sample), and affected individuals who have not been so treated (i.e., the first biological sample). Requirement (iii) is described at page 23, lines 9-13. The same degree of differential expression should not be seen between normal or comparatively insulin sensitive subjects who have been treated with the known effective drug or treatment (i.e., the fourth biological sample), and similar individuals who have not (i.e., the second biological sample). Step (c) of claim 1 has been amended to clarify that it is the cellular tissue of the fifth biological sample which is susceptible to insulin action. Clearly, the recitations of the first, second, third, fourth, and fifth biological samples are inherently described in previously amended claim 1. Specifically, the biological samples recited in the step a) of the previously presented claim 1 are "obtained from insulin resistant, normal or insulin sensitive subjects in response to a known treatment or compound which alters insulin sensitivity". These biological samples are equivalents of the first, second, third, and fourth biological samples recited in currently amended claim 1. The biological sample recited in step b) of the previously amended claim 1 is an equivalent of the fifth biological sample recited in the currently amended claim 1.

Claims 3, 5-7, 14, 15, 16, 18, 22, 24, 25, 29-31, and 33 have been amended in a minor fashion to render the language of the claims consistent with that presented in amended claim 1.

Claim 52 has been amended such that it now depends from the currently amended claim 1.

Claim 53 now depends from claim 1 rather than claim 10.

Claim 4 has been canceled.

No new matter has been introduced by the foregoing amendments. Accordingly, entry of the foregoing claim amendments is respectfully requested.

2. The Restriction Requirement for Claim 29 is Improper:

Applicants note that the Examiner has maintained the restriction requirement relating to election of a single protein encompassed by claim 29 as set forth in the January 26, 2005 Office Action. The Examiner further requests claim 29 be amended to recite the elected protein, LOMT21 only.

Applicants hereby reiterate that this restriction requirement is improper. An important feature of the instantly claimed method is the comparison step of proteins which are already known to possess altered expression levels in response to previously identified treatments which modulate pancreatic islet or β cell function in different test subjects and the expression levels of proteins which are altered in response to contact with the test agent. Comparing the populations of differentially expressed proteins enables the skilled person to identify agents which create more desirable protein expression patterns, i.e., those of a subject having normal pancreatic islet or beta cell function. Thus, it is Applicants' position that any protein which exhibits the foregoing characteristics is encompassed by the claim, thus, the election of a single protein from claim 29 is onerous and improper. It is respectfully requested that the Examiner re-consider this requirement for restriction. Claim 29 will be amended accordingly should the Examiner maintain his position.

3. Claims 1-7, 14-25, 27-32, and 52 as Currently Amended are Fully Enabled by the Specification:

At page 4 of the Office Action, the Examiner has rejected claims 1-7, 14-25, 27-32, and 52 under 35 U.S.C. §112. The Examiner contends that the scope of the present claims includes

an embodiment wherein the sample consists of a subcellular fraction and that such a sample would not be capable of generating a differentially expressed protein pattern when contacted with a test agent.

Applicants respectfully submit that the Examiner's contention is erroneous. It is well known in the art that intact cells are not necessary for protein expression. Naked DNA can be readily transcribed and RNA translated in cell-free systems. Kits for such cell-free expression have been commercially available for many years, if not decades. And, as the Examiner will be well aware, nuclei are not the only organelles in which transcription takes place. Mitochondria also contain nucleic acid and produce a number of their own proteins. Cytosolic fractions contain mRNA and are capable of translating the same into proteins. They can therefore be used to identify agents which exert an effect at the level of translation. Thus subcellular fractions containing (at least) nuclei, cytosol or mitochondria would certainly be suitable for use in the methods of the invention. Indeed, the specification explicitly teaches using urine or a body fluid sample at page 9, lines 30-35.

As the Examiner acknowledges, the level of skill of those working in the field of identifying differentially expressed proteins is high. Such artisans would know which fractions would be suitable for any particular purpose and which would not. Thus the skilled person would be perfectly capable of identifying subcellular fractions which may be used in the methods of the invention.

The Examiner further cites Dreger et al. (Eur. J. Biochem. 270,589-599 (2003)) wherein the analysis of differential protein expression in subcellular fractions was limited to differential expression patterns that were already established prior to the time a cell sample was fractioned. However, it is noted that

Dreger et al. disclose analysis of protein compositions at the level of subcellular structures. Dreger et al., are silent as to the capacity of subcellar fractions and there ability to generate differential protein expression patterns.

In further support of the foregoing, numerous publications involving protein analysis in subcellular fractions in response to test agents are readily available to the skilled person. Two of such publications are enclosed herein to illustrate the principle:

1) Pasquin et al. (Biochem. J. (1980)186,127-133) describe analysis of differential lipid production in isolated rat liver mitochondria and microsomal preparations in the presence and absence of thyroxine (see Table 4). Total protein in each fraction is also measured, to see whether the thyroxine affects protein content of the fraction. A slightly more sophisticated protein analysis could readily be performed by applying proteomic techniques (such as 2D gel electrophoresis) to see if the thyroxine treatment affects the distribution of proteins in each fraction, i.e. results in differential protein expression within a given fraction.
2) Zamzani et al. (J. Exp. Med. (1996)183,1533-1544) report differential release of pro-apoptotic agents from isolated mitochondria treated with agents which affect mitochondrial pore opening. Proteomic analysis of such mitochondria (e.g. by 2D PAGE) before and after treatment would therefore show differential protein content.

Applicant's stand ready to provide the Examiner with several more references in this regard, should the Examiner maintain his position. In light of the above, the skilled practitioner's

ability to determine protein production patterns in subcellular fractions is clearly evident. It is a well settled premise in patent law that a patent need not teach, and preferably omits, that which is well known in the art. Lindemann Maschinenfabrik v. American Hoist and Derrick, 221 USPQ 481, 489 (Fed. Cir. 1984).

It is respectfully submitted that the subject matter of claims 1-7, 14-25, 27-32, and 52 is fully enabled by the disclosure in the specification. Accordingly, the rejection of these claims under 35 U.S.C. §112, first paragraph, is untenable and should be withdrawn.

4. The Meets and Bounds of Claim 15 are Clear to One of Skill in the Art:

At page 8 of the Office Action, the Examiner rejected claim 15 under 35 U.S.C. §112, second paragraph, as allegedly failing to particularly point out and distinctively claim the subject matter regarded as the invention. Specifically, the Examiner contends that it is unclear how insulin sensitve normal control animals can be litter mates of insulin resistant genetically mutated animals. Applicants respectfully submit that Mendalian inheritance patterns of mutated alleles can readily give rise to the situation encompassed by the claims.

The proportion of insulin sensitive and insulin resistant individuals in a particular litter depends on the number and type of mutations involved in causing insulin resistance, and the genotypes of the two parents. For example, assume that insulin resistance is due to mutation of a single gene. If the mutation is dominant, and each parent is heterozygous for that mutation, then 25% of the offspring should be normal and 75% insulin resistant. If only one parent carries the mutation, then 50% of the offspring should be normal and 50% insulin resistant.

Alternatively, if the mutation is recessive and both parents are heterozygous, 75% of the offspring will be normal and 25% insulin resistant. Thus it is perfectly possible for one litter to contain both normal and affected individuals, although the proportions of each type will be dependent on a number of different factors.

Accordingly, it is perfectly possible that normal control animals may have litter mates which are insulin resistant due to genetic mutation. Clearly, claim 15 is definite and its rejection under 35 U.S.C. §112, second paragraph, should be withdrawn.

5. Claims 1-7, 14, 16-20, 22, 23, 33, and 52 as Currently Amended are not Anticipated by either Wang et al. or Stephens et al.:

At page 9 of the Office Action, claims 1-5, 14, 16-20, 22, 33, and 52 stand rejected under 35 U.S.C. §102(b) as allegedly anticipated by Wang et al.(British Journal of Pharmacology (1997)122,1405-1410). Further at page 11, the Examiner rejects claims 1, 6, 7, 18, 22, 23, and 52 under 35 U.S.C. §102(b) as allegedly anticipated by Stephens et al. (Endocrine Reviews Vol. 16, No. 4, 2005). These rejections are improper for the following reasons.

Currently amended claim 1 and claims dependent therefrom recite a method of screening for agents useful to treat insulin resistance. First, a protein marker or markers are identified whose level of expression correlates with insulin sensitivity in a particular model exposed to a known effective treatment (steps (a) and (b) of claim 1). Having identified such a protein or proteins, their expression level is used as a read-out of insulin sensitivity in a screen of candidate agents, to identify further agents which have an effect on insulin sensitivity (steps (c) (d)

of claim 1). Neither Wang nor Stephens disclose such a screen.

Specifically, Wang describes a number of proteins whose plasma levels vary when various animal models are treated with the known insulin-sensitizing drug rosiglitazone. However, it does not suggest that the levels of these proteins can be used as markers in a screen to identify new agents for treating insulin resistance. It certainly does not disclose a method having all the steps of the currently amended claim 1. In addition, in forming the bases of the rejection, the Examiner cites Wang as teaching that rosiglitazone alters the expression levels of NPY, leptin and insulin in insulin-resistant Zucker rats (page 9 of the Office Action). This assertion is incorrect because Wang has clearly stated in its abstract that rosiglitazone has no effect on either plasma leptin levels (point 5) or regional hypothalamic NPY or NPY mRNA levels (points 3 and 4). Thus, not only does Wang fail to teach the steps of identifying marker protein or proteins (as recited in steps (a) and (b) of claim 1), this reference also fails to teach the identification of new agents for treating insulin-resistance using the differentially expressed marker protein or proteins.

The passages in the Stephens references which are cited by the Examiner relate to the mechanism by which GLUT4 glucose transporter molecules circulate between the plasma membrane and intracellular vesicles. This circulation represents a variation in protein location within a cell, but does not necessarily mean that differential expression of the protein is taking place. The document certainly does not suggest that levels of GLUT4 expression (or any other protein) should be used in a screen to indicate whether candidate agents have an effect on insulin sensitivity. And indeed, it does not disclose a screen having all the steps of the currently amended claim 1.

Inasmuch as neither Wang nor Stephens teach all the steps of

the screening methods recited by the present claims, the 35 U.S.C. §102(b) rejections based on these two references is improper and should be withdrawn.

6. Claims 1-7, 14, 16-20, 22, 23, 27-33 and 52 as Currently Amended are Patentable over the Prior Art cited by the Examiner:

At page 13 of the Office Action, the Examiner rejected claims 1-7, 14, 16-20, 22 23, 27-33, and 52 under 35 U.S.C. §103(a) as allegedly being unpatentable over either Wang et al. or Stephens et al. in view of U.S. Patent No. 5,744,300 to Linskens et al. (hereafter, patent '300). This rejection is respectfully traversed for the following reasons.

Patent '300 attempts to identify senescence-related genes by analysis of RNAs expressed differentially between young and senescent cells. It does not analyze differential protein expression. Particularly, Patent '300 is related to methods of identifying senescence-related genes by comparing mRNA levels in senescent cells and young quiescent cells. The cited patent further discloses the use of genetags to quantify differential gene expression in the mRNA levels in response to treatment with active compound. Still disclosed by Patent '300, is the identification of compound which alters gene expression in mRNA levels in senescent cells. In contrast, claim 1 step (b) as amended recites three requirements which a protein must show in order to be used as a marker of insulin sensitivity in a screen for agents capable of treating insulin resistance. Requirement (i) is that the marker is differentially expressed between normal and affected individuals (i.e., between the first and second biological samples). Requirement (ii) is that treatment with a known effective drug affects expression of the marker in affected individuals (i.e., between the first and third biological samples). Requirement (iii) is that treatment of unaffected

individuals with the known drug does not affect expression of the marker, or affects it less than in affected individuals (i.e., between the second and fourth biological samples). This ensures that the effect seen in (ii) is genuinely correlated with insulin sensitivity, and is not a side effect of drug treatment. This analysis ensures that the markers used in the screen are real indicators of insulin sensitivity, is rather more sophisticated than anything disclosed in patent '300, which simply discloses analysis of young cells versus senescent cells. The method steps of claim 1 as amended are not disclosed in patent '300 and cannot be derived from patent '300. Likewise, as discussed above, these steps are not disclosed in either Wang or Stephens. It is a well-settled premise in patent law that "silence in a reference is not a proper substitute for adequate disclosure of facts from which a conclusion of obviousness may justifiably follow". In re Burt, 148 U.S.P.Q. 548 (CCPA 1966)

It is impossible to arrive at the claimed subject matter by combining Wang with patent '300, or by combining Stephens with patent '300. Accordingly, the 35 U.S.C. §103(a) rejection of claims 1-7, 14, 16-20, 22 23, 27-33, and 52 cannot be maintained.

CONCLUSION

In view of the amendments and remarks presented herein, it is respectfully urged that the rejections set forth in the September 16, 2005 Official Action be withdrawn and that this application be passed to issue. In the event the Examiner is not persuaded as to the allowability of any claim, and it appears that any outstanding issues may be resolved through a telephone

interview, the Examiner is requested to telephone the undersigned attorney at the phone number given below.

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Encl.: Pasquin et al. (Biochem. J. (1980)186,127-133; and
Zamzani et al. (J. Exp. Med. (1996)183,1533-1544)

Biochem J. (1980) **186**, 127–133
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Differential Effect of L-Thyroxine on Phospholipid Biosynthesis in Mitochondria and Microsomal Fraction

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1. The action of L-thyroxine on the incorporation of radioactive choline or CDP-choline into phosphatidylcholine *in vitro* was explored in liver and brain microsomal fraction and mitochondria obtained from young adult rats. 2. In liver mitochondria isolated from animals treated with L-thyroxine (40 mg/kg body wt. during 6 days), the incorporation of both radioactive precursors into phosphatidylcholine was significantly decreased compared with normal controls, whereas in the total homogenate and in the microsomal fraction the incorporation was similar in the experimental and control groups. In subcellular fractions isolated from brain, the incorporation of precursors was similar in L-thyroxine-treated and normal animals. 3. Liver mitochondria isolated from normal animals incubated *in vitro* with CDP-choline, in the presence of different concentrations of L-thyroxine, showed also a marked decrease in the incorporation of label into phosphatidylcholine, whereas no significant changes were found in the total homogenate and in the microsomal fraction compared with control experiments. 4. The differential effect of L-thyroxine on the incorporation of radioactive precursors into phosphatidylcholine of isolated liver subcellular fractions gives further support to the hypothesis that liver mitochondria can independently synthesize part of their own phospholipids. 5. Possible mechanisms of the action of the hormone at the mitochondrial level are discussed.

Work from this laboratory (Soto *et al.*, 1972; Krawiec *et al.*, 1975) as well as results presented by Bossman & Case (1969) and by Van Schijndel *et al.* (1973), at variance with results obtained by other investigators (McMurray & Dawson, 1969; Jungalwala & Dawson, 1970; McMurray, 1974), has indicated that both liver mitochondria and microsomal fraction are able to independently synthesize phosphatidylcholine.

Nelson & Cornatzer (1965) showed that the administration of L-thyroxine for 6 days to young adult rats resulted in an increased incorporation of ^{32}P into different phospholipids of liver mitochondria. On the other hand, studying the effects of thyroidectomy on the turnover rate of protein of rat liver mitochondria, Satav *et al.* (1976) concluded that the lack of hormone produced an increase in the half-lives of mitochondrial proteins.

These results, as well as data published by Sterling *et al.* (1977) that indicate a possible direct effect of thyroxine on mitochondria, prompted us to explore the action of thyroid hormone on the incorporation of labelled precursors into phosphatidylcholine of liver and brain mitochondria and microsomal fraction to obtain further support for the hypothesis of an independent synthesis of phosphatidylcholine by mitochondria.

Experimental

Adult Wistar rats of either sex (40–50 days old, 100–120 g body wt.) were used in all studies. To explore the effect of thyroxine *in vivo*, animals were made hyperthyroid as described by Nelson & Cornatzer (1965). L-Thyroxine (sodium salt; kindly supplied by Glaxo Laboratories, Buenos Aires), dissolved in 5 mM-NaOH at a concentration of 4 mg/ml and adjusted to pH 8.1 with HCl, was injected intraperitoneally into each animal once a day (40 mg/kg body wt. per day) for 6 days. Controls were similarly injected with 5 mM-NaOH. On the seventh day, animals were killed by decapitation and brains and livers were rapidly removed. Serum concentrations of L-thyroxine were determined in controls and in experimental animals by radioimmunoassay. All subsequent operations were performed at 0–4°C. Brain and livers were homogenized at 10% (w/v) in ice-cold 0.32 M-sucrose, pH 7.4, by using a Potter–

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Elvehjem-type homogenizer. A portion of the total homogenate was saved for subsequent analysis and the remainder was used for the separation of subcellular fractions as previously described (Soto *et al.*, 1972; Krawiec *et al.*, 1975). Isolated liver and brain mitochondria and microsomal fraction were employed to explore the incorporation of radioactive precursors *in vitro* as described below.

Cross contamination of subcellular fractions

For the detection of microsomal contamination of the mitochondrial fraction we used NADPH–cytochrome *c* reductase, cytochrome *P*-450 and a method that was devised in our laboratories as previously described (Krawiec *et al.*, 1975).

Incubation conditions

Two radioactive precursors (both obtained from New England Nuclear Corp., Boston, MA, U.S.A.) were used for our studies: [*Me*-^{3}H]choline (sp. radioactivity 69.5 Ci/mmol) and CDP-[*Me*-^{14}C]choline (sp. radioactivity 49 mCi/mmol). To study the incorporation of choline, the incubation was done in a medium containing 20 mM-KH_2PO_4/K_2HPO_4 buffer (pH 7.4)/20 mM-sodium pyruvate/1.3 mM-sodium malate/5 mM-$MgCl_2$/1.7 mM-ATP/0.25 mM-CTP/0.1 mM-CoA/1.7 mM-CMP/[*Me*-^{3}H]choline (5 μmol, 0.5 μCi) (McMurray & Dawson, 1969). The final volume was 2 ml. The amount of fraction added to the incubation medium was equivalent to 0.5 g of fresh tissue. For the study of the incorporation of CDP-choline, the medium contained 37.5 mM-Tris/HCl buffer, pH 7.4/10 mM-cysteine hydrochloride (adjusted to pH 7.4 with 0.1 M-NaOH)/20 mM-$MgCl_2$/0.17 M-sucrose/CDP-choline (0.10 μCi, 0.8 μmol) (final volume 2 ml) as described by McMurray & Dawson (1969). The amount of fraction obtained from liver added to the incubation medium was equivalent to 50 mg of fresh-tissue total homogenate or microsomal fraction and to 100 mg of fresh-tissue mitochondria per tube as stated in a previous paper (Krawiec *et al.*, 1975). For the incubation of brain subcellular fractions we used the equivalent of 100 mg of fresh tissue for all fractions.

In the experiments in which we explored the incorporation into liver and brain subcellular fractions obtained from L-thyroxine-treated and control animals, the reaction was started by adding the labelled precursor to the medium containing the fraction and incubation was carried out for 30 min at 37°C with continuous shaking. In the experiments in which we explored the effect of L-thyroxine added *in vitro* on the incorporation of CDP-[*Me*-^{14}C]choline, we used liver and brain total homogenate, mitochondria and microsomal fraction obtained from normal animals. To facilitate the entrance of the hormone, the fractions were first preincubated for 15 min in the presence of three different concentrations of added L-thyroxine: 1 mM, 10 μM and 0.1 μM. The reaction was started by addition of the complete medium and incubation was carried out for 30 min at 37°C with continuous shaking. Appropriate normal controls without L-thyroxine were processed similarly. Incorporation was stopped in all cases by addition of chloroform/methanol (1 : 1, v/v).

Lipid extraction and radioactive counting

Extraction of lipids from the various samples and washing of the extracts was done by the method of McMurray & Dawson (1969). To check that the radioactivity was incorporated into phosphatidylcholine, lipids were separated by t.l.c. as previously described (Seminario *et al.*, 1965) and areas of silica gel corresponding to the various lipids were scraped off the plate and placed in counting vials containing 10 ml of scintillation solution (see below) and 0.7 g of Cab-O-Sil. Of the total radioactivity found in the plate, 90–95% was present in the spot corresponding to phosphatidylcholine. To measure the incorporation of the radioactive precursor, portions of the total lipid extract were evaporated to dryness in counting vials and re-dissolved in 10 ml of a scintillation solution containing 4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis(5-phenyloxazol-2-yl)benzene/litre of toluene.

Chemical and enzymic determinations

ATPase (Mg^{2+}-dependent) activity was measured as described by Richards *et al.* (1977). Proteins were determined by the method of Lowry *et al.* (1951) and lipid phosphorus as described by Chen *et al.* (1956).

The amount of radioactive CDP-choline present in the medium was assessed by extraction of the nucleotide derivative from samples incubated for 30 min with and without subcellular fractions, using the method described by Mandeles & Kammen (1966). Adequate zero-time controls were processed similarly for the purpose of comparison. Briefly, samples were precipitated with an equal volume of cold 20% trichloroacetic acid. After separation of the supernatant, the precipitate was washed once with 10% trichloroacetic acid. Absorbance of the combined supernatants was measured at 260 nm and acid-washed charcoal (Norit) was added in amounts corresponding to 5 mg/unit of $A_{260} \cdot cm^2$ {1 unit of $A_{260} \cdot cm^2 = [A_{260} \cdot \text{volume } (cm^3)]/\text{path length } (cm)$}.

The charcoal-treated material was filtered through a 0.025 mm Millipore filter and the adsorbed nucleotide was eluted by repeated washings with water. Radioactivity present in the water eluate (which corresponds to the CDP-bound radioactive choline) was measured by liquid scintillation by using 10 ml of scintillation solution (Bray, 1960) per 2 ml of the water eluate. Recovery was better than 70% in three different control experiments. Statistical analysis of the results was carried out by the Student's *t* test. A probability level of 0.05 or less was considered a significant difference.

Results

Hormone serum concentrations in treated animals showed values six to seven times higher than those present in normal controls, indicating that L-thyroxine treatment was effective in producing hyperthyroidism. Cross contamination of isolated mitochondria by microsomal fraction, assessed by the different methods mentioned above, was similar in fractions obtained from normal or thyroxine-treated animals and varied between 3 and 7%, depending on the method used (cytochrome *P*-450 gave negative values in most cases).

Table 1 shows that the administration of L-thyroxine to young adult rats during six days produced a 10–20% decrease in body weight and a 10% decrease in liver weight compared with control animals. In L-thyroxine-treated animals there was a statistically significant decrease in the total proteins of liver mitochondria, whereas total protein content in the liver total homogenate and microsomal fraction was not significantly affected in treated animals compared with normal controls. Phospholipid phosphorus on the other hand showed similar results in all fractions in both experimental and control rats.

In animals injected with L-thyroxine the incorporation of radioactive choline into phosphatidylcholine either expressed as nmol/mg of total protein or as nmol/μg of lipid P showed a marked decrease in liver mitochondria in comparison with data obtained in normal animals (Table 2). In the total homogenate and in the microsomal fraction on the contrary, the incorporation was similar in both experimental conditions. Since Nelson & Cornatzer (1965) found that the activity of liver ATPase was significantly increased in animals treated with thyroxine, we decided to investigate the activity of this enzyme in our fractions. A high activity of ATPase could invalidate the results mentioned above, since a decreased availability of ATP in the incubation medium could unspecifically interfere with the incorporation of choline into phosphatidylcholine. Our results (Table 2) shows an increase in the activity of the enzyme of 28% in liver total homogenate, 50% in mitochondria and 41% in microsomal fraction obtained from thyroxine-treated animals compared with controls. Although apparently the decreased incorporation of choline into mitochondria was not due to an increase in the activity of ATPase (the activity of the enzyme was also increased in the total homogenate and specially in microsomal fraction, with no apparent effect on the incorporation of the labelled precursor), we decided to use CDP-choline as precursor in our further experiments, since ATP is not necessary at this stage of the biosynthetic pathway of phosphatidylcholine.

In animals treated with thyroxine, the incorporation of CDP-choline into phosphatidylcholine was significantly decreased in liver mitochondria compared with normal controls. In the total homogenate and in the microsomal fraction, however, the incorporation was similar in experimental and normal animals (Table 2). To ascertain that in hyperthyroid rats there was no increased degradation of CDP-choline by enzymes present in the tissue fractions during incubation, decreasing the availability of CDP-choline in the medium and thus diminishing the synthesis of phosphatidylcholine, radioactive CDP-choline was estimated in control experiments as described in the Experimental section. Results showed that the total nucleotide-bound radioactivity remaining after incubation was quite similar in tubes containing added tissue fraction obtained from hyperthyroid rats and in control tubes containing tissue obtained from normal animals, indicating that the decreased incorporation found in mitochondria was not due to a decreased availability of the labelled precursor, but to other reasons. Results obtained with brain total homogenate, microsomal fraction and mitochondria indicated that the administration of thyroid hormone produced no significant effect on the nervous tissue in adult rats. Brain weight, total protein and phospholipid phosphorus, as well as incorporation of CDP-choline, were similar in both experimental and control animals (Table 3).

In the experiments in which the isolated subcellular fractions were incubated in the presence of thyroxine added to the medium, we also found a decrease in the incorporation of CDP-choline into

Table 1. *Effect of L-thyroxine on total protein and phospholipid content in liver subcellular fractions and on body and liver weight*

Rats (40–50 days old) were injected with L-thyroxine (40 mg/kg body wt.) during 6 days. Liver subcellular fractions were obtained and total protein and phospholipid P were determined as described in the text. Results are means ± S.E.M. for six different experiments. Statistical analysis was carried out by Student's *t* test. A probability level of 0.05 or less was considered a significant difference. Body weight refers to increase in weight during the experimental period (6 days).

	Control	Experimental
Body weight (g)	+17.7 ± 2.0	+7.5 ± 1.9
Liver weight (g)	11.0 ± 0.3	10.0 ± 0.6
Total protein (mg/g of fresh tissue)		
Total homogenate	194.8 ± 17.9	223.8 ± 18.0
Mitochondria	31.6 ± 3.0	23.0 ± 1.6*
Microsomal fraction	27.0 ± 2.5	28.2 ± 2.2
Phospholipid phosphorus (μg/g of fresh tissue)		
Total homogenate	1007.4 ± 62.3	1095.8 ± 91.0
Mitochondria	154.4 ± 14.1	132.0 ± 8.3
Microsomal fraction	240.4 ± 18.1	212.6 ± 12.2

* $P < 0.025$.

Table 2. *Effect of L-thyroxine on the incorporation of radioactive choline and CDP-choline into phosphatidylcholine and on ATPase activity of liver subcellular fractions*

Rats (40–50 days old) were injected with L-thyroxine (40mg/kg body wt.) during 6 days. Liver subcellular fractions were obtained and incubated for 30min in a medium containing the radioactive precursor as described in the Experimental section. Results are means ± S.E.M. for six different experiments. Data of incorporation into mitochondria are those obtained after subtraction of the possible incorporation due to contaminating microsomal fraction, which was estimated as described in the Experimental section. Statistical analysis was carried out by the Student's *t* test. A probability level of 0.05 or less was considered a significant difference. †, $P<0.05$; ‡, $P<0.01$; **, $P<0.02$; *, $P<0.01$.

	Choline incorporation			
	Control		Experimental	
	(nmol/μg of P)	(nmol/mg of protein)	(nmol/μg of P)	(nmol/mg of protein)
Total homogenate	0.24 ± 0.05	1.32 ± 0.22	0.21 ± 0.04	1.45 ± 0.28
Mitochondria	0.23 ± 0.05	1.51 ± 0.13	0.13 ± 0.02†	0.85 ± 0.13‡
Microsomal fraction	0.09 ± 0.02	0.96 ± 0.07	0.11 ± 0.03	1.02 ± 0.08
	CDP-choline incorporation			
Total homogenate	1.10 ± 0.04	4.83 ± 0.30	0.95 ± 0.08	3.85 ± 0.17
Mitochondria	0.28 ± 0.03	1.32 ± 0.27	0.15 ± 0.02**	0.82 ± 0.09†
Microsomal fraction	1.69 ± 0.10	12.23 ± 1.28	1.69 ± 0.08	10.91 ± 0.89

	ATPase activity (nmol of P_i released/min per mg of protein)	
	Control	Experimental
Total homogenate	105.7 ± 9.6	135.2 ± 12.3*
Mitochondria	114.9 ± 11.4	172.3 ± 22.3*
Microsomal fraction	70.9 ± 9.9	100.3 ± 11.6*

Table 3. *Effect of L-thyroxine on the incorporation of radioactive CDP-choline into phosphatidylcholine of brain subcellular fractions*

Rats (40–50 days old) were injected with L-thyroxine (40mg/kg body wt.) during 6 days. Brain subcellular fractions were obtained and incubated in an appropriate medium containing the radioactive precursor as described in the Experimental section. Results are means for six different experiments ± S.E.M.

	Specific radioactivity			
	Control		Experimental	
	(d.p.m./μg of P)	(d.p.m./mg of protein)	(d.p.m./μg of P)	(d.p.m./mg of protein)
Total homogenate	21.6 ± 3.6	249 ± 12.8	18.8 ± 2.1	215 ± 18.7
Mitochondria	89.3 ± 7.5	716 ± 54.5	81.6 ± 7.6	654 ± 55.7
Microsomal fraction	22.9 ± 2.8	291 ± 25.2	22.5 ± 3.5	287 ± 30.7
Myelin	34.1 ± 2.9	941 ± 87.9	40.8 ± 5.2	946 ± 61.5
Nerve endings	28.4 ± 4.2	286 ± 31.2	25.8 ± 3.5	278 ± 19.8

phosphatidylcholine of liver mitochondria at all hormone concentrations, whereas in total homogenate and microsomal fraction there were no differences in specific radioactivity compared with control (Table 4). Total protein, lipid phosphorus and ATPase activity in fractions incubated with the hormone showed no changes relative to controls.

Discussion

Three points are relevant in the discussion of our results: (*a*) the differential effect of thyroxine (in both experimental conditions) on protein content and on incorporation of radioactive CDP-choline, into phosphatidylcholine of isolated liver mitochondria, total homogenate and microsomal fraction; (*b*) the lack of effect of thyroxine on the various parameters analysed in adult (mature) brains; (*c*) the possible mechanisms through which the hormone exerts its action on phosphatidylcholine synthesis at the mitochondrial level.

Enzymes involved in the biosynthesis of phospholipids have been found in the endoplasmic reticulum by Wilgram & Kennedy (1963), but work by McCaman & Cook (1966) in brain and Stoffel &

Table 4. *Effect of added L-thyroxine on the incorporation of radioactive CDP-choline into phosphatidylcholine of liver mitochondria and microsomal fraction*

Liver mitochondria and microsomal fraction were obtained from normal 40–50 day-old rats. The isolated fractions were pre-incubated for 15 min in the presence of different concentrations (1 mM–0.1 μM) of L-thyroxine and further incubated for 30 min in the complete medium containing radioactive CDP-choline. For details see the Experimental section. Results are means ± S.E.M. for six different experiments. Data of incorporation into mitochondria are those obtained after subtraction of the possible incorporation due to contaminating microsomal fraction, which was estimated as described in the Experimental section. Statistical analysis was carried out by Student's *t* test. A probability level of 0.05 or less was considered a significant difference. Abbreviation: N.S., not significant.

	Incorporation into phosphatidylcholine			
	Mitochondria		Microsomal fraction	
	(nmol/μg of P)	(nmol/mg of protein)	(nmol/μg of P)	(nmol/mg of protein)
Control	0.27 ± 0.03	1.13 ± 0.12	1.60 ± 0.12	15.38 ± 1.06
1 mM-Thyroxine	0.15 ± 0.03	0.62 ± 0.12	1.69 ± 0.16	14.99 ± 1.25
	$P < 0.02$	$P < 0.02$	N.S.	N.S.
10 μM-Thyroxine	0.15 ± 0.01	0.67 ± 0.10	1.72 ± 0.14	16.06 ± 1.20
	$P < 0.02$	$P < 0.02$	N.S.	N.S.
0.1 μM-Thyroxine	0.17 ± 0.01	0.67 ± 0.10	1.67 ± 0.16	16.75 ± 1.07
	$P < 0.05$	$P < 0.05$	N.S.	N.S.

Schiefer (1968), Kaiser & Bygrave (1968) and Kaiser (1969) in rat liver suggested that mitochondria can synthesize phospholipids through the CTP-mediated pathway. Work by Jungalwala & Dawson (1970), Miller & Dawson (1972) and McMurray (1974), however, indicated that mitochondria were unable to synthesize their own phospholipids and postulated that the low synthesizing activity found in these organelles was due to microsomal contamination. In previous work we have dealt extensively with this subject and have come to the conclusion that mitochondria have a certain degree of autonomy for the synthesis of phospholipids (Soto *et al.*, 1977). Clear evidence supporting this point of view was presented by Van Schijndel *et al.* (1973).

Although the results obtained with radioactive choline agree with those obtained using CDP-choline, for the reasons mentioned above (see the Results section) we shall only deal in the present discussion with the latter ones. Whatever the final mechanism of action of thyroxine on the synthesis of phosphatidylcholine might be, it is clear from our results that the hormone affects the CTP-mediated pathway in mitochondria, but not in microsomal fraction. The observed phenomenon cannot be due to differences in recovery of mitochondrial and endoplasmic-reticulum membranes in their respective fractions produced by thyroxine treatment, since microsomal contamination of mitochondria, assessed by different methods, is similar in fractions obtained from normal controls and thyroxine-treated animals. Besides, the fact that the differential effect on microsomal fraction and mitochondria is also seen in fractions obtained from normal (untreated) animals, when they are incubated in the presence of different concentrations of thyroxine added to the medium, indicates that possible variations in the purity of the fractions are not the explanation for our observations.

If the synthesis of the phospholipid in mitochondria is due only to microsomal contamination (as proposed by Dawson and his co-workers), one would expect to find a similar effect of the hormone on microsomal fraction and mitochondria. The fact that it produces a marked decrease in the incorporation of labelled precursor into mitochondria in the two experimental conditions used in the present work, having no significant effect on the activity of microsomal fraction, seems to indicate that the two synthesizing systems are different and to a certain extent independent. Thus the results presented here give further support to our previous findings and seem to prove definitely that liver mitochondria have the machinery for the synthesis of phosphatidylcholine, irrespective of the presence of endoplasmic reticulum.

Our data in rats injected with thyroxine agree with those of Nelson & Cornatzer (1965) regarding the gross effect of the hormone on body and liver weight. Decrease in protein content in mitochondria was not reported by these investigators. Regarding the effects of the hormone on phospholipid metabolism and content in liver subcellular fractions, our results differ completely from those reported by these investigators. The data referring to incorporation of labelled precursor could be at variance because (*a*) the precursor used was different and (*b*) we measured the incorporation *in vitro* in the isolated fractions after treatment of the animal with

thyroxine, whereas Nelson & Cornatzer (1965) injected the precursor *in vivo* in the treated animals. The problem nevertheless is that we have been unable to confirm their finding of a decreased content of phospholipids in thyroxine-treated liver mitochondria. Unfortunately we have no valid explanation for this discrepancy. Kaiser & Bygrave (1969) explored the incorporation of choline into phosphatidylcholine of liver mitochondria isolated from animals treated with tri-iodothyronine and liver mitochondria isolated from normal animals but incubated with various concentrations of hormone. The results of the first group of experiments are opposite to those obtained by us, since they found an increase in the incorporation of the precursor by mitochondria instead of a decrease. However, in the second group of experiments the results obtained are coincident with our own. Unfortunately Kaiser & Bygrave (1969) do not make any comments on the discrepancies in the results of their two sets of experiments. There are several differences in the experimental design used by these investigators and by us which might explain the discrepancies mentioned above. The low doses of tri-iodothyronine used by these investigators to treat animals compared with very high doses used by us could be an explanation, since it is apparent from the results of their second group of experiments, that when high doses of tri-iodothyronine are present in the medium, the incorporation of the precursors into phosphatidylcholine is completely inhibited.

Regarding the second point, a large number of reports from different laboratories have shown that the effects of thyroxine on brain are only exerted during maturation (Krawiec *et al.*, 1969). Neonatal hyperthyroidism affects the lipid composition of rat cerebral cortex and cerebellum (Faryna de Raveglia *et al.*, 1972) and treatment of newborn rats with thyroxine markedly decreases all lipid classes in the same tissues (Faryna de Raveglia *et al.*, 1973). There are no reports in the literature, to our knowledge, with reference to the effects of thyroxine and thyroid deprivation on the biosynthesis and turnover of phospholipids in brain subcellular fractions. Although we expected to obtain negative results in the present group of experiments with brain tissue, we are presently studying the action of neonatal hyper- and hypo-thyroidism on phospholipid metabolism in the nervous tissue, which, based on the results presented in this paper, appears to be a promising and interesting line of research.

With reference to the possible mechanism of action of thyroxine on phospholipid metabolism at the mitochondrial level, it must be borne in mind that in spite of the large amount of work devoted to this fundamental subject, the effects of thyroxine at the molecular level are far from being known. Sterling *et al.* (1977) have reviewed the literature on this topic and have also demonstrated the presence of thyroxine-binding sites in mitochondrial inner and outer membranes, supporting previous findings of other investigators that indicate that one of the target elements of thyroxine could be the mitochondrion. A long list of enzyme activities has been shown to be affected (decreased or increased) in liver and in other tissues by thyroxine administration (Wolff & Wolff, 1964). The list of enzymes is so diverse that no common mechanism of action of thyroxine can be recognized.

The decreased incorporation of CDP-choline into phosphatidylcholine in liver mitochondria isolated from hormone-treated animals could be explained by a decrease in the activity of CDP-choline–1,2-diacylglycerol cholinephosphotransferase as a consequence of a reduction in the protein content found in these mitochondria. It would be another of the various enzymes adversely affected by thyroxine. Roncari & Murthy (1975) have shown changes in the activity of liver enzymes related to lipid metabolism in rats treated with thyroxine, which were proved to be due to an altered content of enzyme protein and not to variations in the catalytic activity or to the presence of activators or inhibitors. If the activity of CDP-choline–1,2-diacylglycerol cholinephosphotransferase were similarly decreased by the action of thyroid hormone, this could be a possible explanation for our findings. However, the fact that similar results were obtained in the experiments in which the action of the hormone on normal liver mitochondria was explored *in vitro* tends to preclude this hypothesis, since no decrease in protein content was found in this experimental condition.

Since the activity of diacylglycerol acyltransferase is increased in the liver of animals treated with thyroxine and a greater proportion of diacylglycerol is diverted to the synthesis of triacylglycerol (Young & Lynen, 1969), a decrease in the availability of diacylglycerol (see Glenny & Brindley, 1978) could contribute to the diminished synthesis of phosphatidylcholine. However, the same objections mentioned above apply to this interpretation. It has been reported that thyroxine forms a highly insoluble complex with Mg^{2+} (Wolff & Wolff, 1964). CDP-choline–1,2-diacylglycerol cholinephosphotransferase is a Mg^{2+}-dependent enzyme and the decreased incorporation of CDP-choline into mitochondria found in our experiments could be due to the lack of this bivalent cation. We have carried out a few experiments to explore this possibility and have found that the decreased incorporation into mitochondria was not corrected by the addition of Mg^{2+} to the incubation medium. In fact, the incorporation was further decreased when the Mg^{2+} concentration in the medium was higher than 150 mM. Since Ca^{2+} is a potent inhibitor of phosphatidylcholine syn-

thesis, a change in partition of this cation between mitochondria and medium produced by thyroxine could explain our results. We have performed a set of experiments in which 1 mM-EGTA (final concentration) was present in the solution used for the isolation of subcellular fractions and in the incubation medium and have obtained similar results, ruling out this possibility. Coates *et al.* (1978) showed that the activity of an acid lipase present in liver increases with thyroid treatment. An uneven distribution of this enzyme in the microsomal and mitochondrial fractions due to the action of the hormone might perhaps explain our results. There are several reasons that make this hypothesis unlikely: optimum pH of this enzyme is acidic, whereas our assays are performed at neutral pH; triacylglycerols and cholesteryl esters (not diacylglycerols) are considered to be their substrates; the enzyme is dependent on Ca^{2+}, which is deliberately omitted from the incubation medium. The main argument against this possibility is again that the differential effect of thyroxine is observed with fractions treated *in vitro* with thyroxine, in which no possible uneven distribution could be expected to occur, since they are obtained from normal animals.

Further studies are required to elucidate the possible mechanisms of action of L-thyroxine on phospholipid metabolism in mitochondria.

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Mitochondrial Control of Nuclear Apoptosis

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Summary

Anucleate cells can be induced to undergo programmed cell death (PCD), indicating the existence of a cytoplasmic PCD pathway that functions independently from the nucleus. Cytoplasmic structures including mitochondria have been shown to participate in the control of apoptotic nuclear disintegration. Before cells exhibit common signs of nuclear apoptosis (chromatin condensation and endonuclease-mediated DNA fragmentation), they undergo a reduction of the mitochondrial transmembrane potential ($\Delta\Psi_m$) that may be due to the opening of mitochondrial permeability transition (PT) pores. Here, we present direct evidence indicating that mitochondrial PT constitutes a critical early event of the apoptotic process. In a cell-free system combining purified mitochondria and nuclei, mitochondria undergoing PT suffice to induce chromatin condensation and DNA fragmentation. Induction of PT by pharmacological agents augments the apoptosis-inducing potential of mitochondria. In contrast, prevention of PT by pharmacological agents impedes nuclear apoptosis, both in vitro and in vivo. Mitochondria from hepatocytes or lymphoid cells undergoing apoptosis, but not those from normal cells, induce the disintegration of isolated Hela nuclei. A specific ligand of the mitochondrial adenine nucleotide translocator (ANT), bongkrekic acid, inhibits PT and reduces apoptosis induction by mitochondria in a cell-free system. Moreover, it inhibits the induction of apoptosis in intact cells. Several pieces of evidence suggest that the proto-oncogene product Bcl-2 inhibits apoptosis by preventing mitochondrial PT. First, to inhibit nuclear apoptosis, Bcl-2 must be localized in mitochondrial but not in nuclear membranes. Second, transfection-enforced hyperexpression of Bcl-2 directly abolishes the induction of mitochondrial PT in response to a protonophore, a pro-oxidant, as well as to the ANT ligand atractyloside, correlating with its apoptosis-inhibitory effect. In conclusion, mitochondrial PT appears to be a critical step of the apoptotic cascade.

Since it has been shown that anucleate cells (cytoblasts) can be induced to undergo programmed cell death (PCD)[1] (1–3), it has become clear that a cytoplasmic PCD pathway must function independently from the nucleus. Both mitochondria (4) and specific ced-3–like proteases (5–7) have been accused of participating in the cytoplasmic control of apoptotic nuclear disintegration. We and others (8–12) have recently demonstrated that cells undergo a reduction of the mitochondrial transmembrane potential ($\Delta\Psi_m$) before they exhibit common signs of nuclear apoptosis (chromatin condensation and endonuclease-mediated DNA fragmentation). This applies to different cell types (neurons, fibroblasts, B and T lymphocytes, pre-B cells and thymocytes, myelomonocytic cells) and to different physiological apoptosis inducers (growth factor withdrawal, tumor necrosis factor, ceramide, glucocorticoids, activation-induced cell death, positive and negative selection, irradiation; 8–13). Moreover, these observations extend to pathogen-induced apoptosis, including irradiation-induced PCD (13) and HIV-1–triggered T lymphocyte PCD (14). When PCD is prevented either by genetic manipulations (e.g., p53 loss mutation, bcl-2 hyperexpression) or by pharmacological

[1] *Abbreviations used in this paper:* ANT, adenine nucleotide translocator; Atr, atractyloside; BA, bongkrekic acid; CsA, cyclosporin A; DAPI, 4′-6-diamidino-2-phenylindole dihydrochloride; DEX, dexamethasone; diamide, diazenedicarboxylic acid bis 5*N*,*N*-dimethylamide; $DiOC_6(3)$, 3,3′dihexyloxacarbocyanine iodide; $\Delta\Psi_m$, mitochondrial transmembrane potential; GalN, D-galactosamine; ICE, IL-1β converting enzyme; MCB, monochlorobimane; mCICCP, carbonyl cyanide m-chlorophenylhydrazone; mtDNA, mitochondrial DNA; PCD, programmed cell death; PT, permeability transition; ROS, reactive oxygen species; RR, ruthenium red; *ter*-BHP, *ter*-butylhydroperoxide.

N. Zamzami and S.A. Susin contributed equally to this paper.
This paper is dedicated to José Uriel.

agents (*N*-acetylcysteine, protease inhibitors, linomide), both mitochondrial and nuclear signs of apoptosis are abolished (10, 12, 13). Moreover, cells that have lost their $\Delta\Psi_m$ appear to be irreversibly programmed to die (10). Although these observations suggest the involvement of mitochondria in apoptosis, they do not clarify the cause–effect relationship between mitochondrial dysfunction and subsequent nuclear apoptosis. It appears clear that reactive oxygen species (ROS), which may be generated by uncoupled mitochondria (9, 12), are not essential for the apoptotic process (15–17). Thus, whenever a cause–effect relationship between mitochondrial disorders and nuclear apoptosis exists, it must be mediated by factors other than ROS. The aim of this paper was to unravel the existence of such a pathway linking mitochondrial dysfunction to nuclear disintegration.

As to the mechanism of apoptotic $\Delta\Psi_m$ disruption, pharmacological experiments suggest that it involves the opening of so-called mitochondrial permeability transition (PT) pores (12, 18). Under normal conditions, the inner mitochondrial membrane is quasi-impermeable for small molecules, thus allowing for the creation of the electrochemical gradient which is indispensable for mitochondrial function. However, in determined circumstances, opening of PT pores or "megachannels" allows for the free distribution of solutes of <1,500 daltons and of some proteins, thereby disrupting the $\Delta\Psi_m$ and associated mitochondrial functions (19, 20). In isolated mitochondria, PT is accompanied by colloidosmotic swelling and uncoupling of oxidative phosphorylation, as well as by the loss of low molecular weight matrix molecules such as calcium and glutathione (19–21). It may be important to note that PT is modulated by multiple different physiological and pharmacological inducers and inhibitors (for a review see reference 22) and that PT is both the cause and the consequence of $\Delta\Psi_m$ dissipation, as well as of reactive oxygen metabolite production (19–29). In other terms, PT results ipso facto in $\Delta\Psi_m$ disruption and later in ROS hyperproduction, but $\Delta\Psi_m$ reduction and ROS themselves can also provoke PT, as do many other factors (divalent cations, pH variations, peptides, etc; 22).

The exact molecular composition of the PT pore is not known. However, it appears that at least one inner mitochondrial transmembrane protein, namely the adenine nucleotide translocator (ANT), is involved in PT pore formation (for reviews see references 19, 20) and that ANT associates with several molecules of the outer mitochondrial membrane such as the peripheral benzodiazepine receptor and the voltage-dependent anion channel (30). ANT ligands such as atractyloside (Atr) and bongkrekic acid (BA) enhance or reduce the probability of PT, respectively (31–35).

Based on these premises, we have tested the hypothesis that PT might be the critical event determining the apoptosis-inducing potential of mitochondria. Using a cell- and cytosol-free system in which purified mitochondria and nuclei are confronted, we show that induction of PT by the ANT ligand Atr or other less specific PT inducers causes isolated mitochondria to trigger nuclear apoptosis. In contrast, inhibition of PT by the Atr antagonist BA, as well as by a variety of additional PT inhibitors, abolishes mitochondria-mediated nuclear apoptosis. The apoptosis-inhibitory proto-oncogene product Bcl-2 functions as an endogenous inhibitor of mitochondrial PT. These data establish mitochondrial PT as a critical event of apoptosis.

Materials and Methods

Animals and In Vivo Treatments. Male 6–10-wk-old BALB/c mice were injected simultaneously with D-galactosamine (GalN; 10 mg i.p.) and/or LPS from *Escherichia coli* (Signa Chemical Co., St. Louis, MO; 50 μg i.v.), 5 h before removal of the liver (36). Alternatively, splenocytes were recovered from BALB/c mice 12 h after injection of 1 mg i.p. dexamethasone (DEX; Sigma Chemical Co.) in 200 μl PBS or PBS alone (10, 37).

Cell Lines and In Vitro Culture Conditions. U937 cells were depleted from mitochondrial DNA (mtDNA) by continuous ethidium bromide selection for 4 mo (15). Control experiments revealed that such cells become resistant to antimycin A, which blocks the mtDNA-encoded complex III. Moreover, no mtDNA could be detected by PCR (not shown). 2B4.11 T cell hybridoma cell lines stably transfected with an SFFV.neo vector containing the human *bcl-2* gene or the neomycin (Neo) resistance gene only (38, 39) were kindly provided by Jonathan Ashwell (National Institutes of Health, Bethesda, MD). Cells were cultured in RPMI-1640 medium containing 5% FCS. Apoptosis was induced by culturing cells in the presence of the indicated concentration of diazenedicarboxylic acid bis 5*N*,*N*-dimethylamide (diamide) or carbonyl cyanide m-chlorophenylhydrazone (mClCCP; both from Sigma Chemical Co.). DNA fragmentation of non- or γ-irradiated (10 Gy) thymocytes (10^6 cells/lane) was monitored after culturing cells for 4 h in the presence of DEX (1 μM), etoposide (10 μM; Sigma Chemical Co.), and/or BA (50 μM; purified as described in reference 40), kindly provided by Dr. J.A. Duine (Delft University, Delft, The Netherlands).

Cell-free System of Apoptosis. Nuclei from HeLa or 2B4.11 cells were purified on a sucrose gradient, as described (41), and were resuspended in CFS buffer (220 nM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM PO_4H_2K, 0.5 mM EGTA, 2 mM Cl_2Mg, 5 mM pyruvate, 0.1 mM PMSF, 2 mM ATP, 50 μg/ml creatine phosphokinase, 10 mM phosphocreatine, 1 mM dithiothreitol, and 10 mM Hepes-NaOH, pH 7.4; reagents from Sigma Chemical Co.). Nuclei were conserved at −20°C in 50% glycerol for up to 8 d as described (41, 42). Mitochondria were purified from BALB/c mouse livers, splenocytes, or U937 cells on a Percoll gradient (43) and were stored on ice in B buffer (400 mM mannitol, 10 mM PO_4H_2K, 5 mg/ml BSA, and 50 mM Tris-HCl, pH 7.2) for up to 4 h. For quantitation of nuclear apoptosis, both nuclei (5,000 *g*, 5 min) and mitochondria (2×10^4 *g*, 3 min) were spun down and washed twice in CFS buffer before being mixed. In standard conditions, mitochondria (500 ng/μl protein final concentration) were cultured at 37°C for 90 min with 10^3 nuclei per μl CFS containing a number of different agents: AlF_3, (20 μM), Atr (5 mM; Sigma Chemical Co.), BA (50 μM), $CaCl_2$ (500 μM), mClCCP (10 μM), diamide (100 μM), cyclosporin A (CsA, 10 μM; Sandoz AG, Basel, Switzerland), *N*-methylVal-4-CsA (SDZ 220-384, 10 μM; kindly provided by Dr. Roland Wenger, Sandoz), monochlorobimane (MCB; 30 μM), phosphotyrosine (P; 10 mM), ruthenium red (RR, 100 μM; Sigma Chemical Co.), *ter*-butylhydroperoxide (*ter*-BHP, 50 μM; Sigma Chemical Co.), $ZnCl_2$ (1 mM), AcYVAD-CHO (IL-1β converting enzyme [ICE] inhibitor I), AcYVAD-chloromethylketone (ICE inhibitor II), and/or AcDEVD-CHO (inhibitor of CPP32/

Ced3/Yama; Bachem, Basel, Switzerland). Nuclei were stained with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI; 10 μM) and examined by fluorescence microscopy (5), or were analyzed by agarose gel electrophoresis (10^6 nuclei/lane) (44).

Cytofluorometric Analysis. For $\Delta\Psi_m$ determinations, isolated mitochondria were incubated for 15 min at 37°C in the presence of $DiOC_6(3)$ (80 nM) (45), followed by addition of mClCCP (50 μM), BA (50 μM) and/or Atr (5 mM), and recording of the fluorescence in an Elite cytofluorometer (Coulter Corp., Hialeah, FL) 5 min later. Loss of nuclear DNA (hypoploidy) was determined by propidium iodine staining of ethanol-fixed cells, as described (46).

Large Amplitude Swelling of Isolated Mitochondria. Large amplitude swelling is a colloidosmotic process that is observed among isolated mitochondria undergoing PT in solutions containing low protein concentrations (22). For determination of swelling, mitochondria were washed and resuspended in B buffer (100 μg protein/10 μl buffer), followed by addition of 90 μM CFS buffer and recording of adsorption at 540 nm in a spectrophotometer (model DU 7400; Beckman Instruments, Inc., Fullerton, CA), as described (26). The loss of absorption induced by 5 mM Atr within 5 min was considered 100% the value of large amplitude swelling.

Characterization of Factors Contained in the Supernatant of Mitochondria. Hepatic mitochondria (1 mg/ml in CFS buffer) were left untreated or were incubated with Atr (5 mM) for 10 min at room temperature, followed by ultracentrifugation (1.5×10^5 *g*, 30 min, 4°C). Supernatants were either left untreated or centrifuged through a Centricon 10 membrane (Amicon Inc., Beverly, MA) to separate proteins with an approximate molecular mass of > and <10 kD, following the manufacturers' recommendation. The fraction >10 kD was reconstituted with CFS to the original volume. Supernatants (50% volume) were mixed with nuclei (10^3/μl) in the presence or absence of various antioxidants (50 μM *N-t*-butyl-α-phenylnitrone, 230 μM trolox, 600 μM L-ascorbate, or 1 mg/ml catalase; Sigma Chemical Co.), and nuclei were stained with DAPI after 90 min of culture at 37°C.

Results and Discussion

Isolated Mitochondria Undergoing PT Induce Nuclear Apoptosis in a Cell-free System. One of the PT pore constituents is the ANT. The ANT adopts different molecular conformations when exposed to two specific ligands, Atr and BA (31, 47). Atr favors the opening of the PT pore, whereas BA reduces the probability of PT pore gating (31–35, 47). We have tested the capacity of purified mitochondria with open and closed PT pores to induce nuclear apoptosis in a modified cell-free system (4, 42). Mitochondria were purified from the murine liver and were mixed with Hela nuclei in an isotonic buffer containing an ATP regeneration system. On their own, unmanipulated mitochondria derived from healthy cells are incapable of inducing signs of nuclear apoptosis such as chromatin condensation and endonuclease-mediated DNA fragmentation. However, mitochondria exposed to a dose of Atr that causes PT, determined either as large amplitude swelling (Fig. 1 *A*) or as disruption of the $\Delta\Psi_m$ (Fig 1 *B*), do induce nuclear apoptosis (Fig. 1 *C*) in a time- and dose-dependent fashion (Fig. 1 *D*). Atr does not induce apoptosis itself; it only favors the induction of nuclear apoptosis when mitochondria are present (Fig. 1 *C*). In the presence of BA, mitochondria fail to undergo PT in response to Atr (Fig. 1, *A* and *B*) and lose the capacity to induce chromatin condensation (Fig. 1 *C*) and associated oligonucleosomal DNA fragmentation (Fig.

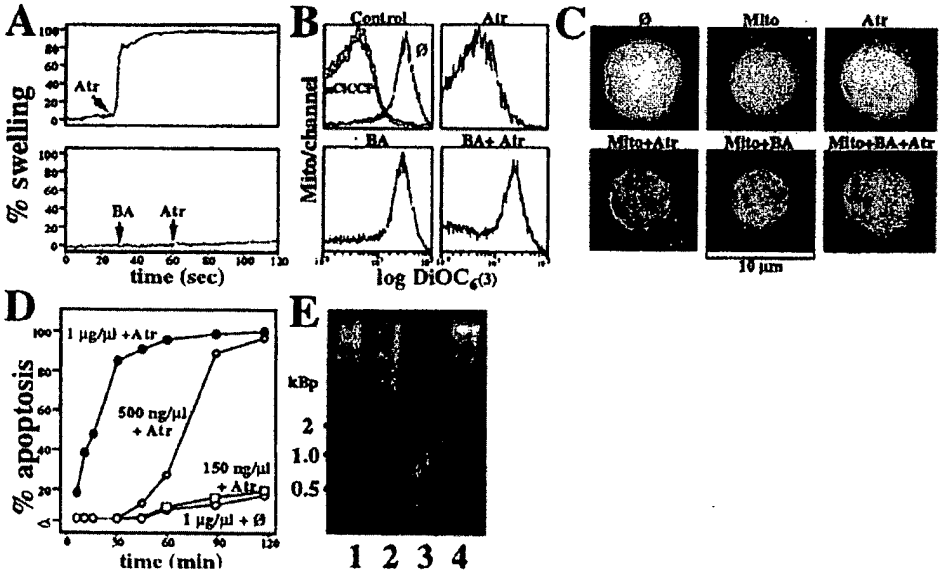

Figure 1. Regulation of mitochondrial PT and mitochondria-mediated nuclear apoptosis by two adenine nucleotide translocator ligands. (*A*) Effect of atractyloside (*Atr*) and bongkrekic acid (*BA*) on mitochondrial large amplitude swelling. (*Arrows*) Time points at which BA (50 μM) and/or Atr (5 mM) were added to mitochondria. (*B*) $\Delta\Psi_m$ of Atr- and BA-treated mitochondria, as determined by incorporation of $DiOC_6(3)$ into mitochondria, 5 min after addition of Atr and/or BA. Incubation with the protonophor mClCCP, which completely disrupts the $\Delta\Psi_m$, unravels $DiOC_6(3)$ background fluorescence. (*C*) Chromatin distribution of isolated HeLa nuclei cultured with mitochondria, Atr, and/or BA for 90 min. Single nuclei stained with DAPI representing the dominant phenotype (≥80%) are shown. (*D*) Time and dose dependence of nuclear chromatin condensation. Nuclei were incubated during the indicated interval with the specified amount of mitochondria (doses in μg protein) and/or Atr. (*E*) Fragmentation of nuclear DNA induced by mitochondria. Purified HeLa nuclei were cocultured with mitochondria (500 ng/μl), Atr, and/or BA for 90 min (same conditions as in *C*), followed by agarose gel electrophoresis of ethidium bromide–stained DNA. Lane *1*: Atr only; lane *2*: mitochondria only; lane *3*: mitochondria plus Atr; and lane 4: mitochondria plus Atr plus BA.

1 *E*). These findings suggest that PT controls the apoptosis-inducing capacity of mitochondria.

Mitochondria Lacking mtDNA Can Undergo PT and Cause Nuclear Apoptosis in a Cell-free System. Although most proteins contained in mitochondria are encoded by nuclear genes, a number of proteins including some components of the respiratory chain complexes I, III, and IV, are encoded by the mitochondrial genome. Previously, it has been reported that cell lines lacking mtDNA can undergo full-blown nuclear apoptosis (15), and this finding could give rise to the interpretation that mitochondria are not important for the control of apoptosis. To challenge this (over)interpretation, we purified mitochondria from cells lacking mtDNA (ρ° cells), as well as from control ρ^+ cells, and tested their apoptosis-inducing potential. As shown in Fig. 2 *A*, ρ° mitochondria can undergo large amplitude swelling in response to Atr, exactly as do ρ^+ control organelles. Moreover, in the presence of Atr, ρ° mitochondria are as efficient inducers of nuclear disintegration, as are control ρ^+ mitochondria (Fig. 2 *B*). This indicates that all mitochondrial functions critical for apoptosis induction are encoded by nuclear genes. Thus, in accord with the published data (15), the capacity of mitochondria to induce nuclear apoptosis does not depend on the presence of mtDNA. Furthermore, the fact that respiration-deficient ρ° mitochondria (which may be expected to produce less ROS than ρ^+ control organelles) conserve their proapoptotic activity, suggests that ROS do not mediate apoptosis in this cell-free system.

Strict Correlation Between PT-associated Swelling and Nuclear Apoptosis Induction. The above results suggest that mitochondria are indeed efficient inducers of nuclear apoptosis, provided that they are undergoing PT. This conclusion is corroborated by the strict correlation between PT and the proapoptotic effect of mitochondria, when PT is induced by a variety of different molecules: Atr, the pro-oxidant *ter*-BHP, and calcium ions via the thiol–cross-linking agent diamide or the protonophore mClCCP (Fig.3). All these reagents are thought to act via distinct mechanisms: Atr by virtue of its capacity to interact with the ANT (31–35); calcium via conformational effects on proteins that are yet poorly understood (19, 20, 22); hydroperoxides via oxidation of mitochondral glutathione and pyridine nucleotides (48); diamide via its thiol–cross-linking action on the ANT(49, 50); and protonophores via dissipation of the proton gradient ($\Delta\Psi_m$), then entailing PT as a secondary phenomenon (24, 27, 28). Control experiments indicate that none of these reagents induces nuclear apoptosis by itself, i.e., in the absence of mitochondria (not shown). Atr-induced swelling and nuclear apoptosis are efficiently inhibited by BA and CsA, as well as by the thiol reagent MCB. CsA can be substituted for by the nonimmunosuppressive CsA analogue *N*-methylVal-4-CsA (51), indicating that its PT and apoptosis-inhibitory effect is not mediated via calcineurin. A series of substances previously reported to inhibit apoptosis in a cell-free system (4) can also inhibit both PT and mitochondria-mediated nuclear apoptosis: phosphotyrosine, $ZnCl_2$, and AlF_3 (Fig. 3). Other substances (4) have no or little (<20%) inhibitory effects on Atr-induced swelling and apoptosis: calpain inhibitors I and II, GTPγS, and ionomycin. Similarly, synthetic tetrapeptide inhibitors of ICE and of CPP32/Yama fail to interfere with PT and PT-dependent nuclear apoptosis (Table 1). Calcium-driven but not Atr-induced PT and apoptosis are selectively inhibited by RR, a specific inhibitor of the mitochondrial calcium uniport (52), underscoring the fact that proapoptotic calcium effects are indeed mediated by mitochondria. As expected (50), the syn-9,10-dioxa-bimanine halogen derivative MCB is particularly effective in inhibiting PT and apoptosis induced by diamide (Fig. 3). In accord with published data (for a review see reference 22), none of the inhibitors used in this study is capable of providing long-term (>30 min) protection against mitochondrial swelling in response to the whole panel of PT inducers. This probably reflects the profound differences in the molecular mechanisms of PT caused by different inducers (19, 20, 22, 24, 27, 28, 48–50).

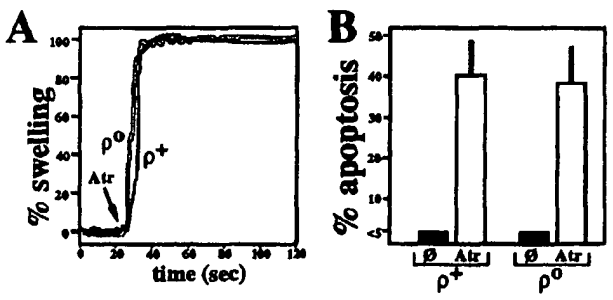

Figure 2. Mitochondrial swelling and nuclear apoptosis induction do not require mitochondrial DNA. (*A*) Large amplitude swelling of mitochondria lacking mtDNA. Mitochondria obtained from normal U937 cells (ρ^+) or U937 cells lacking mtDNA (ρ°) were incubated while assessing absorbance at 540 nM. (*Arrow*) Addition of Atr (5 mM). (*B*) Induction of nuclear apoptosis by mitochondria lacking mtDNA. HeLa nuclei were cultured for 90 min with Atr and/or mitochondria obtained from ρ^+ or ρ° U937 cells, followed by determination of the frequency of apoptotic or damaged nuclei.

In synthesis, the strict correlation existing between mitochondrial PT and mitochondria-mediated nuclear apoptosis suggests that PT is indeed a crucial event in the regulation of apoptosis induction by mitochondria. Moreover, the fact that none of the inhibitory substances (BA, CsA, MCB, RR, phosphotyrosine, $ZnCl_2$, AlF_3) suppresses PT and apoptosis in response to all PT inducers (Fig. 3) suggests that they do not directly affect nuclei but rather act via PT modulation. Finally, the data summarized in Fig. 3 underscore the complex pharmacology of mitochondrial PT.

Mitochondria from Cells Undergoing Apoptosis Transfer Nuclear Apoptosis to a Cell-free System. According to several studies (8–10, 12), mitochondrial function is perturbed early during the apoptotic process. Accordingly, mitochondria isolated from hepatocytes exposed in vivo to an apoptosis-inducing combination of GalN and LPS (36, 53), but not control cells treated with GalN or LPS only, display a reduced uptake of the cationic lipophilic dye 3,3′ dihexyloxacarbocyanine iodide ($DiOC_6[3]$) (Fig. 4 *A*), indicating a

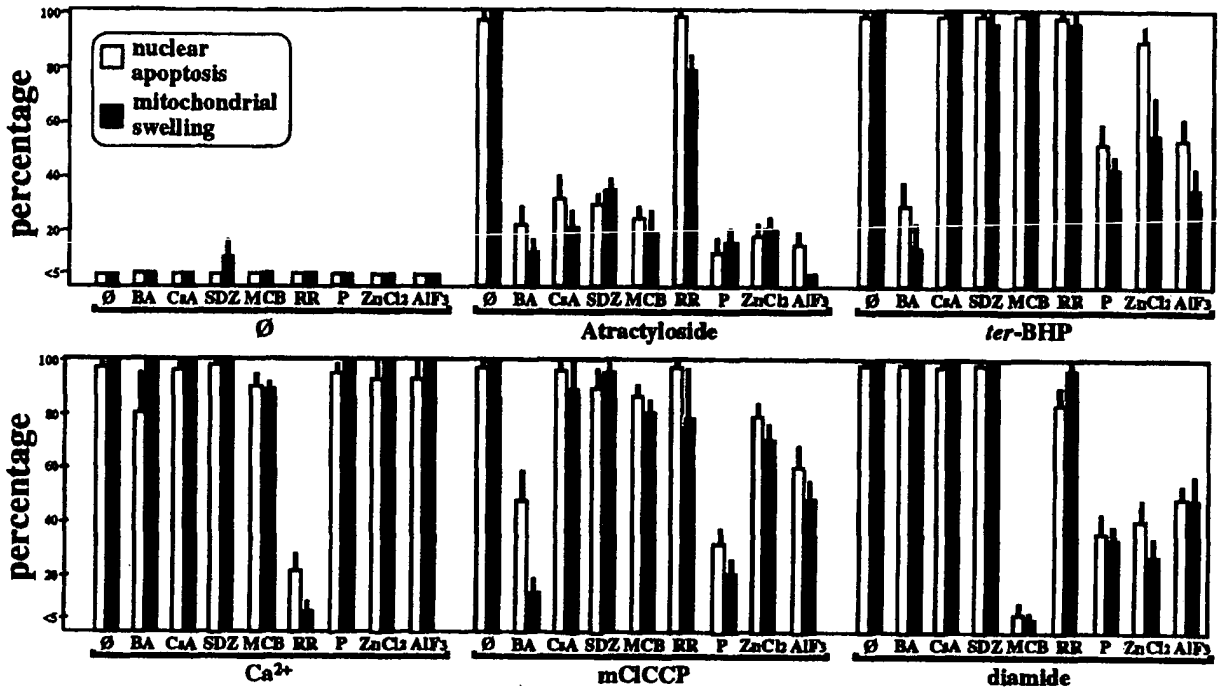

Figure 3. Correlation between mitochondrial swelling and mitochondrial induction of nuclear apoptosis. Mitochondria from hepatocytes were incubated with the PT inducers Atr (5 mM), *ter*-BHP (50 μM), $CaCl_2$ (500 μM), mClCCP (10 μM), or diamide (100 μM) and/or the PT inhibitors BA (50 μM), CsA (10μM), *N*-methylVal-4-CsA (*SDZ*; 10 μM), MCB (30 μM), or RR (100 μM). Mitochondria were also incubated with P (10 mM), $ZnCl_2$ (1mM), or AlF_3 (20 μM). The percentage of condensed nuclei cocultured with mitochondria was recorded after 90 min of incubation at 37°C (*open columns*, X ± SEM of triplicates). Large amplitude swelling was recorded after 5–90 min of culture (*black columns*). Data are shown for 60 min, when the correlation between nuclear apoptosis and mitochondrial swelling is optimal. These results are representative of five independent experiments.

decrease of the $\Delta\Psi_m$. Such mitochondria from apoptotic liver cells cause nuclear apoptosis in vitro (Fig. 4 *B*). Similarly a fraction of mitochondria from splenocytes treated in vivo with the glucocorticoid analogue DEX (10, 37) display a reduced $\Delta\Psi_m$ (Fig. 5 *A*) and cause apoptosis of isolated Hela nuclei in vitro (Fig. 5 *B*). Thus, mitochondria from different cell types undergoing apoptosis in vivo are endowed with the capacity of apoptosis induction in a cell-free system.

Inhibition of PT Inhibits Nuclear Apoptosis both In Vitro and In Vivo. The ANT ligand BA is the agent with the broadest PT-inhibitory spectrum among all substances tested thus far (Figs. 1 and 3) and is the only PT inhibitor that is truly specific for a mitochondrial structure. We

Table 1. *Substances that Fail to Modulate PT and Mitochondria-dependent Nuclear Apoptosis*

Substance	Dose range	Inhibition of PT	Inhibition of nuclear apoptosis
Calpain inhibitor I	100 μM–1 mM	None*	None
Calpain inhibitor II	100 μM–1 mM	None	None
ICE inhibitor I	100 μM–500 μM	None	None
ICE inhibitor II	100 μM–500 μM	None	None
AcDEVD-CHO	100 μM–500 μM	None	None
GTPγS	100 μM–1 mM	None	None
Ionomycin	10 μM–100 μM	None	None

*The indicated substances were employed to modulate the induction of PT in isolated mitochondria induced by the following reagents: Atr, *ter*-BHP, calcium, m-ClCCP, and diamide (same conditions and concentrations as in Fig. 3). Absence of inhibition indicates <20% suppression of either mitochondrial swelling (measured at 60 min as in Fig. 3) or nuclear condensation (measured at 90 min in the same conditions as in Fig. 3), in response to all tested PT inducers.

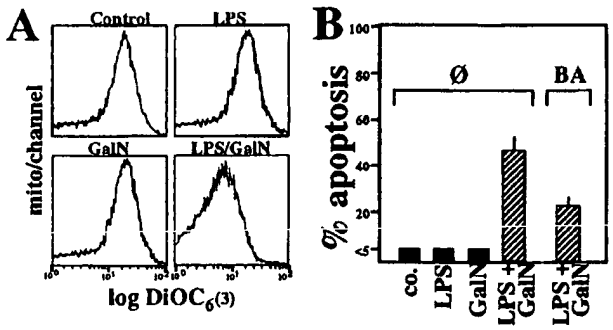

Figure 4. Mitochondria from apoptotic hepatocytes cause nuclear apoptosis in a cell-free system. (*A*) Reduction of the $\Delta\Psi_m$ in mitochondria from cells undergoing apoptosis in vivo. Liver mitochondria were obtained from animals treated with an apoptosis-inducing combination of GalN and/or LPS, followed by $DiOC_6(3)$ labeling for $\Delta\Psi_m$ assessment. (*B*) Mitochondria from apoptotic hepatocytes induce nuclear apoptosis. After the indicated in vivo treatment, liver mitochondria were purified and added to HeLa nuclei in the presence or absence of BA (50 μM), followed by evaluation of chromatin condensation as in Fig. 1. Data are representative of two independent experiments.

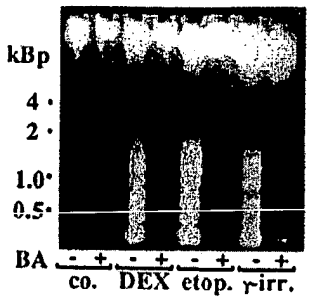

Figure 6. BA inhibits apoptosis of thymocytes induced by various stimuli. Murine thymocytes were exposed to DEX (1 μM), etoposide (10 μM), or cultured after γ-irradiation (10 Gy) in the presence or absence of BA (50 μM). DNA fragmentation of thymocytes (10^6 cells/lane) was monitored after a 4-h culture period. Results are representative of three independent experiments.

therefore tested the effect of BA on the mitochondria-mediated transfer of apoptosis from whole cells undergoing PCD to the cell-free system. As shown in Figs. 4 *A* and 5 *A*, BA partially reduces the proapoptotic effect of mitochondria from GalN/LPS–stimulated hepatocytes of DEX-primed splenocytes in vitro. This inhibition is significant: 48 ± 10% for GalN/LPS–treated hepatocyte mitochondria and 44 ± 9% for DEX-primed splenocyte mitochondria. In addition, BA is highly efficient (>90% inhibition) in preventing the death of intact thymocytes exposed to a series of different apoptosis inducers: DEX, irradiation, and topoisomerase inhibition (Fig. 6). These results corroborate the notion that mitochondria are indeed involved in the apoptotic cascade in vivo and that mitochondrial PT is both sufficient and necessary to induce nuclear apoptosis.

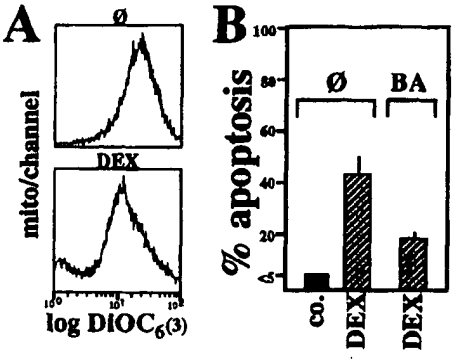

Figure 5. Transfer of apoptosis by mitochondria from apoptotic splenocytes. (*A*) $\Delta\Psi_m$ disruption of mitochondria from DEX-primed splenocytes. Mitochondria were purified from control splenocytes (*co.*) or from splenocytes exposed to the glucocorticoid DEX in vivo, followed by staining with $DiOC_6(3)$. (*B*) Mitochondria from apoptotic splenic lymphocytes induce nuclear apoptosis. Mitochondria from control or DEX-primed splenocytes were mixed with HeLa nuclei either in the absence or in the presence of BA to assess their apoptosis-inducing potential as in Fig. 4 *B*. Splenic mitochondria were tested at a concentration of 250 ng/μl. Typical results out of three independent experiments are shown.

Bcl-2 Inhibits Apoptosis by Preventing Mitochondrial PT. The proto-oncogene product Bcl-2 inhibits apoptosis in response to a number of different stimuli (for a review see reference 54) and prevents both the mitochondrial and the nuclear manifestations of apoptosis (12). Bcl-2 is localized in the mitochondrial outer membrane and endoplasmatic reticulum, as well as in nuclear membranes (55–57). Within the mitochondrion, it is found at the inner–outer membrane contact site, where PT pores are expected to form (20). To map the antiapoptotic function of Bcl-2 either to mitochondria or to nuclei, we purified these organelles from hBcl-2–transfected murine T cell hybridoma cells (39), as well as from mock-transfected controls. Reconstitution experiments indicate that Atr-treated mitochondria from hBcl-2–transfected cells fail to provoke nuclear apoptosis (Fig. 7 *A*) in conditions in which mitochondria from vector-transfected cells (Fig. 7 *A*) or from hepatocytes constitutively lacking Bcl-2 expression (Figs. 1 and 3) do induce nuclear apoptosis. In contrast, nuclei from hBcl-2–transfected cells readily condense and fragment in the presence of Atr and control mitochondria (Fig. 7, *A* and *B*). Thus, in accord with previous genetic (55, 57) and functional (4) studies, the mitochondrial but not the nuclear localization of Bcl-2 is critical for its antiapoptotic function. In control experiments, mixtures of Bcl-2–transfected and control mitochondria induce apoptosis (Fig. 7 *A*), indicating that the Bcl-2–mediated inhibition of apoptosis acts in *cis* and cannot be attributed to cytosolic Bcl-2 contaminating the mitochondrial preparation. In addition, isolated mitochondria from Bcl-2–transfected cells are protected against Atr-induced PT, i.e., they fail to undergo large amplitude swelling and $\Delta\Psi_m$ disruption in response to Atr (Fig. 7, *C* and *D*). Bcl-2 is a potent inhibitor of some death pathways, including pro-oxidants (58), but is comparatively inefficient in preventing calcium-induced and antigen receptor–mediated PCD (38, 39, 59). We therefore tested whether Bcl-2 would be a universal inhibitor of PT or rather, whether it would have a selective effect. As shown in Fig. 8 *A*, Bcl-2 prevents large amplitude swelling of isolated mitochondria in response to M-ClCCP and *ter*-BHP, but not in response to calcium or diamide. These data underscore that different PT inducers obey different mechanisms; this is also suggested by experiments involving PT inhibitors

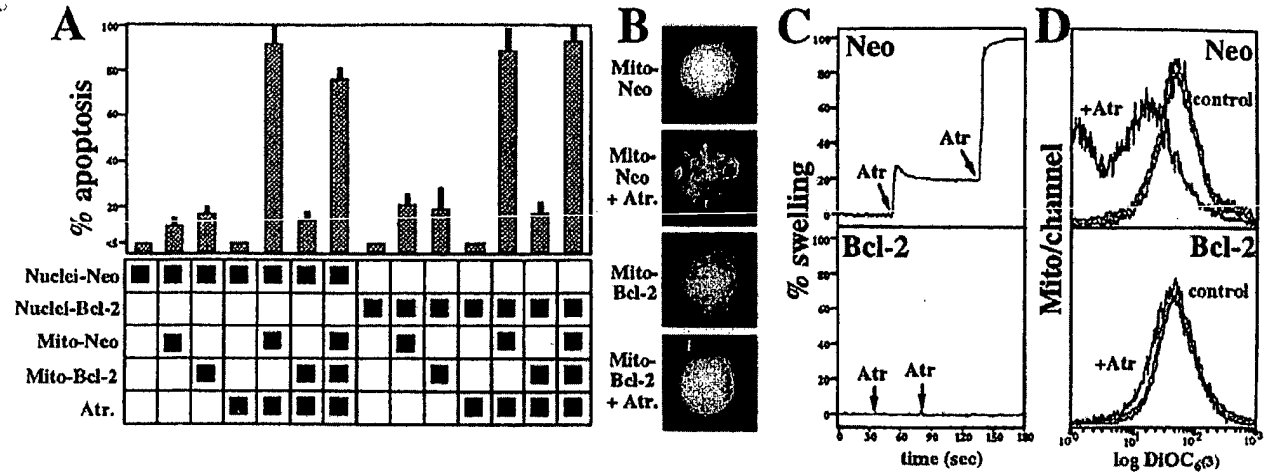

Figure 7. Mechanism of the antiapoptotic effect of Bcl-2. (*A*) Functional mapping of the site at which Bcl-2 acts to prevent Atr-induced apoptosis. Nuclei and mitochondria from Bcl-2– or Neo-transfected cells were cocultured in the presence or absence of Atr (5 mM), as indicated by black squares. After 90 min of coculture, nuclei were stained with DAPI and analyzed for apoptotic morphology. (*B*) Representative nuclei from Bcl-2–transfected cells incubated with the indicated type of mitochondria and/or Atr (same experiment as *A*). (*C*) Bcl-2 directly inhibits the Atr-induced large amplitude swelling of mitochondria. Mitochondria from Neo– or Bcl-2–transfected cells were monitored for large amplitude swelling (as in Fig. 1 *A*). (*Arrows*) Repeated addition of 2.5 mM Atr (final concentration 5 mM). (*D*) Bcl-2 inhibits the Atr-induced disruption of the mitochondrial transmembrane potential. Mitochondria were labeled with $DiOC_6(3)$, cultured for 5 min in the presence or absence of 5 mM Atr, and were then analyzed by cytofluorometry.

(Fig. 3). The pattern of the bcl-2 effect corresponds most closely to that of BA, i.e., it inhibits PT induced by Atr (Figs. 3 and 7), m-ClCCP, and *ter*-BHP, but not calcium or diamide (Figs. 3 and 8). Again, as in the case of BA, Bcl-2–mediated inhibition of PT results in the abolition of the apoptotic potential of isolated mitochondria. More importantly, the Bcl-2–driven inhibition of mitochondrial swelling (Fig. 8 *A*) correlates with its apoptosis-inhibitory potential in cells. Bcl-2 protects against apoptosis of T cell hybridoma cells induced by m-ClCCP (Fig. 8 *B*) and oxidants such as H_2O_2 (58), yet fails to confer protection against diamide (Fig. 8 *B*) and CD3 cross-linking (12, 38, 39). Thus, Bcl-2 does not prevent apoptosis when death is induced via such agents as diamide (Fig. 8 *B*) against whose PT-inducing potential it does not protect (Fig. 8 *A*). Again, these data are in accord with the hypothesis that Bcl-2 prevents apoptosis by virtue of its PT-inhibitory potential.

A Soluble Factor Released from Mitochondria Undergoing PT Mediates Nuclear Disintegration. As shown above, mitochondria undergoing PT induce apoptotic nuclear disintegration in a cell-free system. Whereas some authors have shown that mitochondria are necessary to induce apoptosis in cell-free systems (4, 60), others have found that cytosolic (organelle-free) extracts may be sufficient to induce nuclear

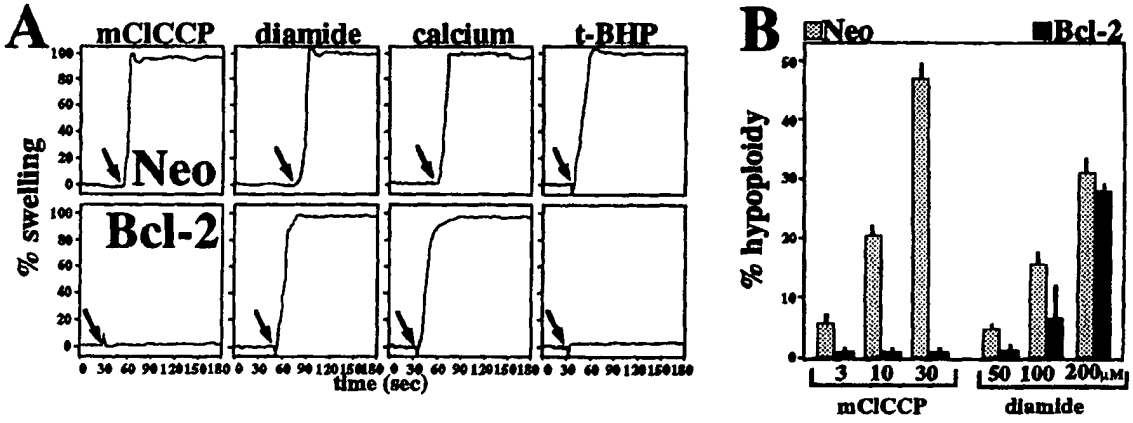

Figure 8. Correlation of the antiapoptotic and the PT-inhibitory effect of Bcl-2. (*A*) Effect of Bcl-2 on large amplitude swelling of isolated mitochondria. (*Arrows*) The indicated reagents were added (same concentrations as in Fig. 3), while absorbance at 540 nm was monitored. (*B*) Spectrum of Bcl-2–mediated inhibition of apoptosis in whole cells. Bcl-2, or Neo-transfected T cell hybridoma cells were cultured with the indicated dose of mClCCP pr diamide for 6 or 24 h, respectively. The percentage of cells with nuclear hypoploidy was determined after ethanol fixation and staining with propidium iodine.

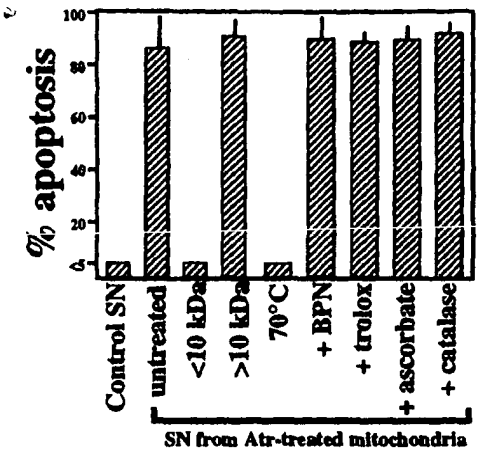

Figure 9. Partial characterization of a proapoptotic activity released from Atr-treated mitochondria. Liver mitochondria were incubated in the presence or absence of 5 mM Atr, followed by ultracentrifugation (150,000 *g*, 30 min). Isolated HeLa nuclei were incubated in the presence of this supernatant to determine the frequency of cells exhibiting apoptotic morphology (90 min, 37°C). Supernatants were heat treated (70°C, 5 min) or centrifuged through membranes with a molecular mass exclusion of ~10 kD before the test. Alternatively, the antioxidants *N-t*-butyl-α-phenylnitrone (50 μM), trolox (230 μM), L-ascorbate (600 μM), or catalase (1 mg/ml) were added to the assay.

apoptosis in vitro (5, 42). Prompted by this apparent contradiction, we tested whether mitochondria undergoing PT would release a soluble proapoptotic factor. As shown in Fig. 9, mitochondria treated with Atr release (a) soluble factor(s) into the supernatant (150,000 *g*, 30 min) that can induce chromatin condensation in isolated HeLa nuclei. This activity is heat sensitive (70°C, 5 min), has a molecular mass >10 kD, and is not neutralized by antioxidants such as *N-t*-butyl-α-phenylnitrone or the water-soluble vitamine E analogue trolox (Fig. 9). In conclusion, at least part of the apoptotic activity of mitochondria is mediated by one or several proteins and does not involve ROS. PT-dependent release of proteins from mitochondria has been reported previously (61).

Concluding Remarks

As shown in this article, mitochondria from hepatic, myelomonocytic, or lymphoid cells induce nuclear apoptosis, provided that they undergo PT. Modulation of PT determines the apoptosis-inducing effect of mitochondria in a cell-free system. Moreover, inhibition of PT by BA, a specific ligand of one PT pore constituent, reduces naturally occurring apoptosis, and Bcl-2 apparently functions as an endogenous PT inhibitor. Although these findings establish mitochondrial PT as a critical event in early apoptosis, they do not resolve a number of issues concerning the cellular biology of apoptosis.

According to studies performed in *Caenorhabditis elegans*, at least two gene products, ced-3, which encodes a cysteine protease, and ced-4, whose function is unknown, are required for apoptosis to occur (62). At present, the sequence of events that eventually link ced-3–like proteases and ced-4 to mitochondria remains unknown. At present, it appears clear that both Bcl-2 (which controls PT; Figs. 7 and 8) and protease activation control two checkpoints of the apoptotic cascade (63). Tetrapeptide inhibitors of the ced-3 homologue CPP32/Yama and of ICE fail to interfere with the induction of PT in isolated mitochondria. Moreover, they fail to inhibit the mitochondria-mediated induction of nuclear apoptosis (Table 1). When thymocyte apoptosis is induced by Fas/CD95 cross-linking, inhibition of ICE prevents both the nuclear manifestations of apoptosis and the $\Delta\Psi_m$ disruption (Marchetti, P., and G. Kroemer, unpublished results). This may indicate that at least some of the members of the family of ced-3–like proteases regulate events that are upstream of mitochondria. At present, however, our data cannot distinguish between two alternative possibilities. First, the PT and the protease-regulated checkpoints of the apoptotic effector phase could be placed in a serial (hierarchical) fashion. Second, both protease activation and PT could form part of parallel pathways culminating in nuclear apoptosis.

It remains largely unknown how Bcl-2 regulates PT on the molecular level. Bcl-2 does not prevent PT as such; it prevents the induction of PT by determined stimuli such as Atr, mClCCP, and *ter*-BHP, but not calcium or diamide (Figs. 7 and 8). Bcl-2 could act via direct molecular association with constituents of the PT pore, a possibility that is suggested by the localization of both Bcl-2 and PT pore constituents at inner–outer membrane contact sites (55–57). Alternatively, Bcl-2 could affect PT indirectly. Thus, it enhances oxidative phosphorylation (64) and causes mitochondrial inner membrane hyperpolarization (65), which in turn would reduce the probability of PT (24). It has previously been reported that mitochondrial membrane localization is necessary to mediate Bcl-2 suppression of apoptosis, namely when apoptosis is induced by E1B-defective adenovirus (57) and when it is triggered by IL-3 starvation of IL-3–dependent 32D cells (55). In contrast, in some other systems of apoptosis induction, a mutated Bcl-2 molecule lacking the membrane localization domain (4, 58), as well as the naturally occurring apoptosis-inhibitory Bcl-2 analogue Bcl-XΔTM (a splice variant of Bcl-X that lacks the transmembrane domain; 66), maintain their antiapoptotic potential. However, the fact that soluble, ubiquitous Bcl-2 still maintains at least part of a its antiapoptotic function does not formally exclude that it acts on the external membrane of mitochondria. The present data suggest an intimate linkage between Bcl-2 and mitochondrial regulation. In this context it may be intriguing that the *C. elegans bcl-2* homologue, *ced-9*, is an element of a polycistronic locus that also contains *cyt-1*, a gene that encodes a protein similar to cytochrome b560 of the mitochondrial respiratory chain complex II (67). Thus both functional and genetic evidence link Bcl-2 to mitochondrial regulation. Irrespective of the exact molecular mechanism by which Bcl-2 affects PT, the finding that Bcl-2 does inhibit PT, at least in response to certain stimuli (Figs. 7 and 8), provides an explanation for hitherto apparently contradictory reports.

Bcl-2 hyperexpression has been reported to inhibit the production and/or adverse effects of ROS (58, 68), that in turn, however, are not obligatory for apoptosis (16). In accord with these findings, Bcl-2 prevents oxidant-mediated PT (Fig. 8). Moreover, it prevents the mitochondrial ROS formation that is secondary to PT (12). Thus, Bcl-2 impedes PT as well as two dissociable consequences of PT: (*a*) nuclear apoptosis, and (*b*) mitochondrial uncoupling and superoxide anion generation.

A further issue that remains to be elucidated is the molecular mechanism by which isolated mitochondria undergoing PT cause nuclear chromatin condensation and endonuclease activation. It appears clear that this mechanism is neither cell type nor species specific, given that, for example, mouse liver mitochondria in PT can promote the apoptotic disintegration of nuclei purified from human fibroblast-like nuclei (Fig. 1). Our data indicate that mitochondria contain or are associated with (a) pre-formed soluble mediator(s) >10kD that is/are released after PT and that alone is/are sufficient to cause nuclear apoptosis (Fig. 9). In accord with published experiments performed on intact cells (16, 17), antioxidants do not neutralize this apoptosis inducer (Fig. 9). Thus, ROS that are formed by mitochondria after PT do not participate in the induction of nuclear apoptosis; this is also indicated by experiments involving ρ° cells that lack a functional respiratory chain (15, and Fig. 2). Moreover, it appears improbable that Ced-3–like proteases would be responsible for this apoptosis-inducing activity, given that the mammalian Ced-3 analogue CPP32 per se is not sufficient to induce nuclear apoptosis in a cell-free system (6). Thus, the molecular events linking mitochondrial PT to nuclear apoptosis await further characterization.

From the available data, it appears that $\Delta\Psi_m$ disruption, which presumably is mediated by PT, is a constant feature of early apoptosis (8–14). Indirect biochemical evidence has previously accused PT to participate in the postischemic or toxin-mediated death of myocardial cells and hepatocytes (69–72), thus again suggesting that PT is a general regulator of cell death. Indeed, the PT pore is an attractive candidate for a death switch that, once activated, marks a point of no return in PCD. At least six reasons support this concept. First, as shown here, PT is both necessary and sufficient to cause nuclear apoptosis. Second, opening of PT pores entails multiple potentially lethal alterations of mitochondrial function (loss of $\Delta\Psi_m$, uncoupling of the respiratory chain, hypergeneration of ROS, and loss of mitochondrial glutathione and calcium; 12, 19–21) and thus may initiate pleiotropic death pathways. Moreover, as shown here, PT triggers a nuclear apoptosis effector pathway whose biochemical components remain elusive. Third, the PT pore functions as a sensor for multiple physiological effectors (divalent cations, ATP, ADP, NAD, $\Delta\Psi_m$, pH, thiols, and peptides), thereby integrating information on the electrophysiological, redox, and metabolic state of the cell (19, 20, 73, and Fig. 3). Thus, different death inducers can converge at this level. Fourth, given that a PT pore constituent such as the ANT is essential for energy metabolism, mutations in this apoptosis-regulatory device will be mostly lethal for the cell. In teleological terms, this would have the advantage of precluding apoptosis-inhibitory (oncogenic) mutations at this level of the apoptotic cascade. Fifth, at least one of the PT constituents, the ANT, is encoded by several members of a gene family that are expressed in a strictly tissue-specific manner (74). Thus, PT pores may be regulated in each cell type in a slightly different fashion. Sixth, PT is endowed with self-amplificatory properties in the sense that loss of matrix Ca^{2+} and glutathione, depolarization of the inner membrane, and increased oxidation of thiols, that result from PT pore opening, all increase the PT pore-gating potential (19–21, 23–29). The self-amplificatory property of PT is also underscored by the data presented in this paper. Thus, induction of PT induces $\Delta\Psi_m$ disruption (Figs. 1 and 7) and, conversely, $\Delta\Psi_m$ depolarization by mClCCP causes PT, measured as large amplitude swelling (Figs. 3 and 8). Similarly, oxidant treatment causes PT (Figs. 3 and 8), and PT will ultimately entail mitochondrial generation of ROS (12). The fact that some consequences of PT (e.g., $\Delta\Psi_m$ dissipation, ROS generation) themselves may cause PT suggests that PT may engage in a positive feedback loop that contributes to apoptotic autodestruction. Thus, PT would have to respond in an all-or-nothing fashion and, once activated, would seal the cell's fate in an irreversible fashion. Accordingly, cells exhibiting an immediate consequence of PT, that is $\Delta\Psi_m$ reduction, are irreversibly committed to cell death (10).

Apart from these theoretical considerations, the current data suggest that the PT pore occupies a central position in apoptosis regulation. It therefore becomes an attractive target for regulation by pharmacological agents, as well as by endogenous apoptosis regulators belonging to the ever-expanding Bcl-2 gene family.

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